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Production of Hydroxyphaseollin in Soybean Leaves Infected with the Leaf Blight Bacterium, *Xanthomonas phaseoli* var. *sojae* and Its Antifungal Action*

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Summary

Hydroxyphaseollin, a phytoalexin of soybean, was detected in soybean leaves infected with the leaf blight bacterium, *Xanthomonas phaseoli* var. *sojae*. Quantities of crude hydroxyphaseollin in infected leaves were proportional to the number of lesions per leaf. Spore germination, specially germ-tube growth of *Fusarium solani* f. *pisi* was inhibited when cultured in the solution containing crude hydroxyphaseollin. Using thin-layer chromatography (TLC) with hexane: ethyl acetate: methanol (60: 40: 1, v/v) as solvent, hydroxyphaseollin and a related compound were detected at Rf of 0.43 and 0.30, respectively. When the TLC plates were inoculated with *Colletotrichum gloeosporioides* zones of inhibition occured in areas corresponding to Rf of 0.43 and 0.30, and germ-tube growth of this fungus was inhibited by elutions from these two zones on the TLC plate.

Introduction

The production of phytoalexin in soybeans, *Glycine max* (L) Merr., that is one of the antifungal metabolites, was reported first by Uehara,[24,25] and Nonaka *et al.*[18] also isolated a similar antifungal principle from soybean pods inoculated with spore suspension of *Fusarium solani* f. *pisi*. Subsequently, antifungal agents have been isolated from soybeans by a number of investigators.[2,6,8,10,19,20] In particular Klarman and his co-workers[12~16,21] have made a thorough study, using various treatments, on phytoalexin production in the soybean. These workers obtained evidence that a pterocarpan related to phaseollin,[3] the phytoalexin from *Phaseollus vulgaris*, was produced by heavy metals or fungus challenged cotyledons and hypocotyls of soybean plants. Gray, Klarman and Bridge[7] measured the relative quantities of antifungal metabolites produced in resistant and susceptible plants inoculated with the soybean pathogen and demonstrated that the resistant plants produced significantly more antifungal metabolites than the suceptible ones when inoculated with the pathogen.

The chemical characters of soybean phytoalexin were clarified by Keen et al.[9] The structure of the soybean phytoalexin molecule was determined by Sims et al[22] and it has been named hydroxyphaseollin (HP) because it has a similar molecular structure to phaseollin. This compound had absorption peaks at 288, 293 and 315 nm.[16] Recently, Keen and Kennedy[11] demonstrated that hydroxyphaseollin and the related isoflavanoids accumulated on soybean leaves inoculated with *Pseudomonas glycinea*.

The objectives of the present investigation were to detect hydroxyphaseollin in soybean

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leaves infected with leaf blight bacterium, *Xanthomonas phaseoli* var. *sojae*, and examine its antifungal properties.

I. Isolation of crude hydroxyphaseollin from soybean leaves infected with *X. phaseoli* var. *sojae*. and its inhibition of spore germination

Materials and methods. Soybean (variety: Tamanishiki) leaves infected naturally with bacterial leaf blight disease caused by *X. phaseoli* var. *sojae* were collected. Extract of crude hydroxyphaseollin from the infected soybean leaves was prepared by a modification of a technique described by Keen et al[9] (Fig. 1). The final volume of extract was

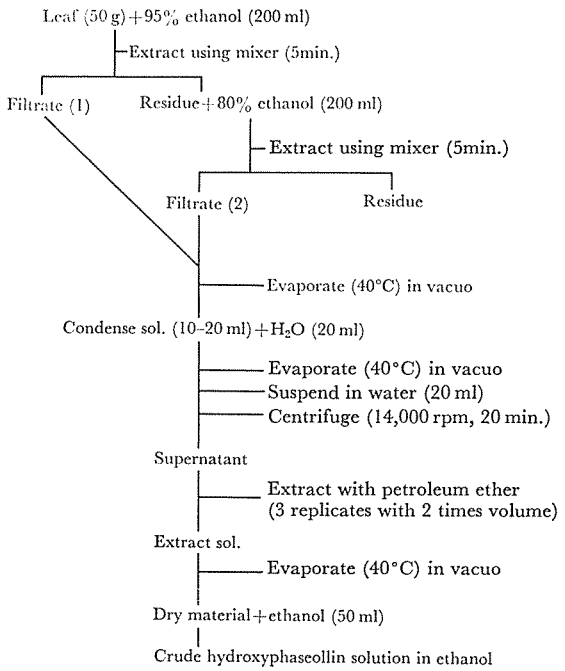

Fig. 1. Extraction procedures of crude hydroxyphaseollin from soybean leaves infected with *Xanthomonas phaseoli* var. *sojae*.

adjusted to one m*l* of ethanol solution per gram of fresh weight of infected soybean leaves. Crude hydroxyphaseollin in the extract was detected by comparing its ultraviolet absorption spectrum with that of hydroxyphaseollin reported by Klarman et al.[16] A Hitachi 139 Type Spectrophotometer was used for this purpose. The bioassay of crude hydroxyphaseollin was carried out at a number of dilution prepared as follows. Ten millilitres of crude HP in ethanol was evaporated in vacuo at 40°C. This was dissolved in 2 m*l* of a solution consisting of 1.97 m*l* of Czapek liquid medium containing 3% ethanol and 0.03 m*l* of ethanol. This gave a solution that was 5 times as concentrated as the original HP solution. Solutions that were 2.5 times and 1.25 times as concentrated as the original HP solution were obtained by appropriate dilutions with alcoholic Czapek media. The inhibitory effects of these solutions on spore germination and germ-tube growth of *Fusarium solani* f. *pisi* were compared with those from extracts of healthy leaves obtained by the same technique.

Results. Maximum absorbance of the extract from infected soybean leaves was obtained at 288 and 315 nm, and minimum absorbance was at 260 nm as shown in Fig. 2. This spectrum resembles that of hydroxyphaseollin reported by Klarman et al.[16] Furthermore no such a specific spectrum was found in the extract from healthy plants.

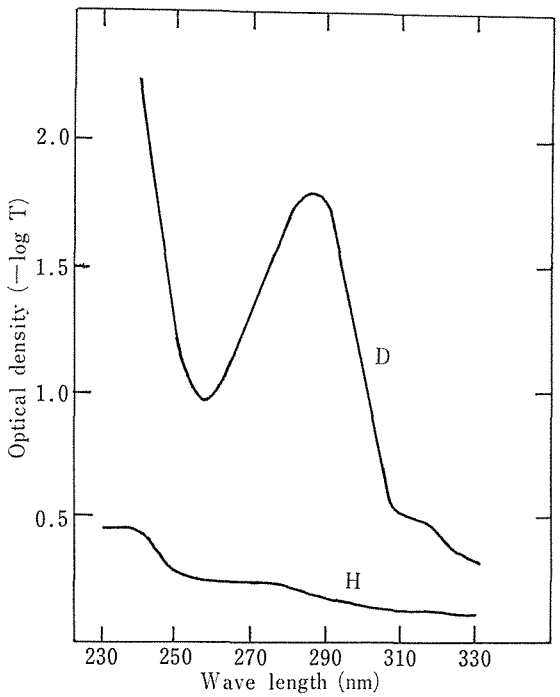

Fig. 2. The ultraviolet absorption spectra of crude extract from soybean leaves infected with *X. phaseoli* var. *sojensis* (D) and from healthy soybean leaves (H).

Table 1. Inhibitory action of crude extracts from soybean leaves infected with *X. phaseoli* var. *sojae* against the spore germination of *Fusarium solani* f. *pisi*.

Extract	Concentration	Spore counted	Germination rate (%)	Germ-tube length
				μ %***
Infected	0*	557	86.7	87.3 (100)
	1.25**	551	78.2	34.7 (39.7)
	2.50**	486	64.3	21.5 (24.6)
	5.00**	549	41.5	15.1 (17.2)
Healthy	0*	1,133	95.7	111.9 (100)
	1.25**	1,239	97.8	96.7 (86.5)
	2.50**	1,141	97.5	87.1 (77.9)
	5.00**	1,154	97.8	96.6 (86.4)

* 3% ethanol solution as control. All solutions tested contain 3% ethanol.

** The figures of 1.25, 2.50 and 5.00 show times in condensation against original extract solution (1 ml/1 g as fresh weight) from infected and healthy leaves, respectively.

*** Figures in parenthesis show per cent of each of germ-tube length in each concentration solutions to that in control solution.

Spore germinations were inhibited by extracts of infected leaves and, especially germ-tube growth was severely retarded in high concentrations of crude hydroxyphaseollin as shown in Table 1. Germ-tube length at 5 times of the concentration of the original HP solution was only 17.2% of the length of those grown in extracts from healthy plants. No inhibition of spore germination was observed in the extracts from healthy plants.

II. Relationship between numbers of lesion on infected leaves and concentrations of crude hydroxyphaseollin

Materials and methods. Soybean leaves of uniform size and infected with bacterial leaf blight were divided into the following three grades of infection according to the average number of lesion per leaf: infection grade 1 was a slight infection having 90 lesions per leaf, infection grade 2 was a moderate infection having 450 lesions per leaf and infection grade 3 was a severe infection having 850 lesions per leaf. The lesions on leaves in the severe infection grade did not coalesce and were separated by sufficient uninfected tissue to permit accurate counting. Extracts were prepared from 50 g (fresh weight) of leaves in each case and the crude hydroxyphaseollin was determined spectrophotometrically as previously described.

Results. The experimental results show (Fig. 3) that the quantity of crude hydroxyphaseollin is proportional to the number of lesions per leaf as long as they are separated

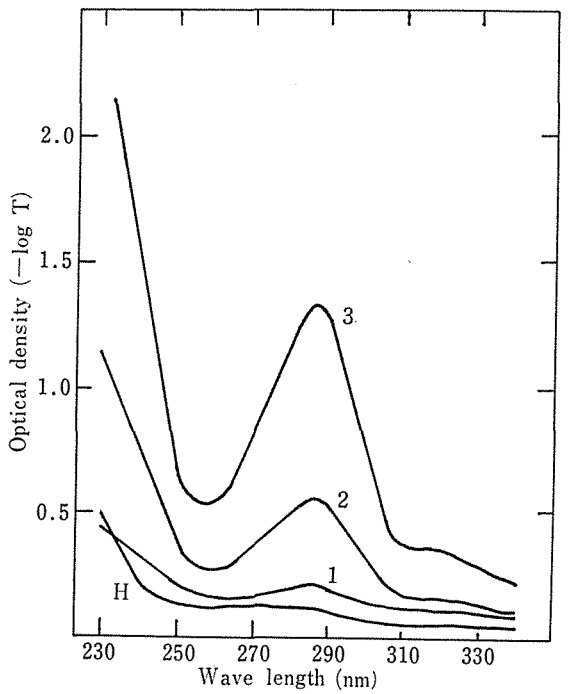

Fig. 3. The ultraviolet absorption spectra of crude extracts from infected soybean leaves divided into three kinds of infection grade in severity.

Infection grades were shown as grade 1 (Fig. 3; 1), 2 (Fig. 3; 2) and 3 (Fig. 3; 3). Grade 1, 2 and 3 had 90, 450 and 850 lesions per leaf in average, respectively. H showed the spectrum of crude extract from healthy soybean leaves.

by sufficient uninfected tissue. Also, in another experiment crude hydroxyphaseollin was detected in infected petioles on which necrotic lesions were formed by the infection of *X. phaseoli* var. *sojae*.

III. The inhibitory effect of purified hydroxyphaseollin on spore germination

1. Antifungal bioassay of hydroxyphaseollin.

Materials and methods. An ethanol solution of crude hydroxyphaseollin extracted from 10 g of infected soybean leaves as previously described was evaporated to dryness with a rotary evaporator at 40°C. The residue in the evaporating flask was dissolved in one m*l* of ethyl acetate.

A thin-layer chromatography (TLC) bioassay technique modified from Klarman and Sanford[16] was used to detect the antifungal principles, hydroxyphaseollin and related compounds. Glass plates 20×20 cm coated with a 0.5 mm layer of sillica gel G (E. Merck, Darmstadt, Germany) were used as TLC plates. After the ethyl acetate solution containing hydroxyphaseollin was spotted on the plates with glass capillary tubes, the plates were developed with the following solvent system: hexane, ethyl acetate and methanol (60: 40: 1, v/v). The solvents were completely evaporated from the plates following their development. The bioassay organism, *Colletotrichum gloeosporioides* Penzig isolated from Satsuma orange, was grown in test tubes containing potato sucrose agar (PSA).

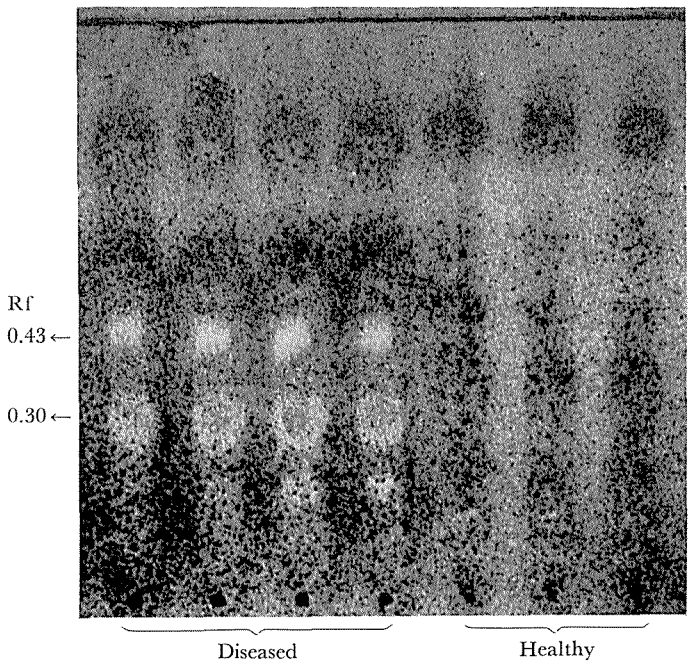

Fig. 4. Thin-layer chromatographic bioassay of extracts from soybean leaves infected with *Xanthomonas phaseoli* var. *sojae*. Extracts were spotted and the plate developed with hexane: ethyl acetate: methanol (60: 40: 1, v/v) before spraying with *Colletorichum gloesporioides* spores. Light areas denote antifungal activity; dark areas denote stimulation of fungus growth. Left in the plate shows bioassay of extract from diseased leaves and right shows that of healthy ones.

Spore suspension of the fungus was passed through cheese cloth, placed into an atomizer and delivered to the surface of the TLC plates in a fine mist. These inoculated plates were then sprayed with PSA at about 45°C and incubated in a moist chamber for 72 hours at 25°C. The spores germinated on the plate surface causing it to become black and the position of the antifungal material could be determined by white zones of adsorbosil in those places where spore germination was inhibited. Therefore, the zones where inhibition occured on the plates appeared as white zones on a dark background.

Results. By means of the chromatographic bioassay (Fig. 4), we were able to detect several inhibitory zones in the extract from infected soybean leaf that were not present in that from healthy plants. The greatest inhibition was observed in zones which corresponded to Rf values of 0.30 and 0.43 on the TLC plates. In addition fungal growth was slightly inhibited in areas which corresponded to Rf values of 0.30, 0.60 and 0.85, respectively.

2. Purification of hydroxyphaseollin by TLC

Materials and methods. Ethyl acetate solutions of extracts from soybean leaves were chromatographed on TLC plates as described above. Silica gel from zones with Rf values of 0.30 and 0.43, corresponding to the inhibition zones (Fig. 4), were scrapped from the plates using a razor. The inhibitory principles were eluted from the scrappings with 10 m*l* of ethanol. These elutions were filtered in order to remove silica gel and the

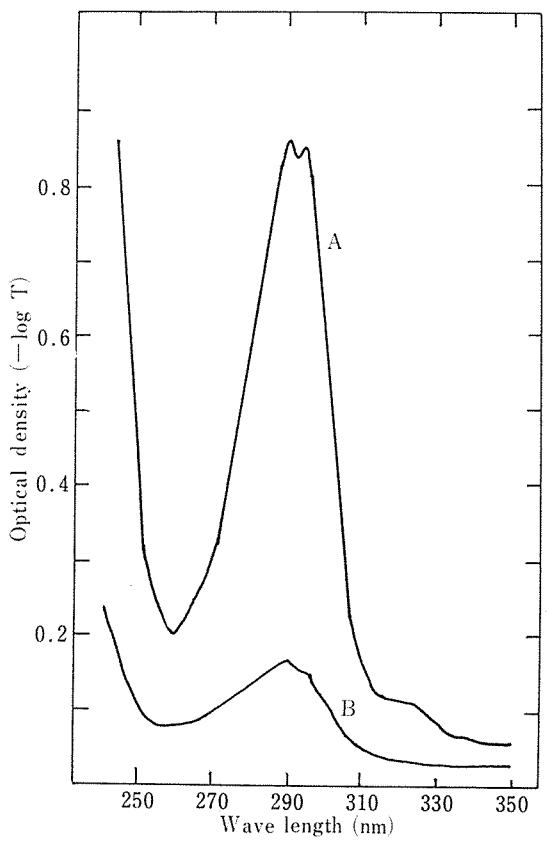

Fig. 5. Ultraviolet absorption spectra of the elutions from the spots of Rf 0.45 (A) and 0.30 (B) on TLC plate, respectively.

ultraviolet absorption spectrum of the inhibitory principle was determined spectrophotometrically as previously described.

Results. The ultraviolet absorption spectra of the antifungal principles produced by soybean leaves infected with *X. phaseoli* var. *sojae* are shown in Fig. 5. Absorbance maxima of the eluate from the position of Rf 0.43 were 288, 293 and 320 nm. This spectrum is characteristic of hydroxyphaseollin. Absorbance maxima of the eluate from the Rf 0.30 zone were 288 and 293 nm and this spectrum also resembles that of hydroxyphaseollin.

3. Antifungal bioassay of purified hydroxyphaseollin

Materials and methods. Purified hydroxyphaseollin and the related compound from both positions at Rf of 0.43 and 0.30 on the TLC separations of extracts of diseased soybean leaves, respectively, were bioassayed using the techniques described above.

Results. No significant differences were observed in the ability of both compounds to inhibit spore germination as compared with the control. Both compounds inhibited the growth of spore germ-tube. The compound eluted from the Rf 0.43 position had a greater inhibitory effect than did that from the Rf 0.30 position (Table 2).

Table 2. Inhibitory action of hydroxyphaseollin and the related compound purified by TLC against the spore germination of *Colletotrichum gloeosporioides*.

Extract	Elution from spot on plate (Rf)	spore counted*	spore germinated*	Germ-tube length**
				(μ)
Infected	0.30 (related compound)	542 (100)***	478 (88.3)	30.2 (29.6)
	0.43 (hydroxyphaseollin)	546 (100)	503 (92.0)	18.5 (17.4)
Healthy	0.43	516 (100)	504 (97.6)	106.5 (100)

* Total spore number obtained by four replicates.

** Mean value of germ-tube length of 500 spores.

*** Figures in parenthesis show per cent of each of germination rate and germ-tube length in each of extract solutions from infected leaves to that in extract solution from healthy ones.

Discussion

Uehara[24,25] reported that a phytoalexin was induced in soybean pods inoculated with *Fusarium* sp., and Nonaka et al[18] also isolated a similar antifungal principle from fungal infected soybean pods. Furthermore, Klarman and Gerdemann[12,13] demonstrated that a phytoalexin was produced by inoculation of soybean seedling hypocotyls with *Phytophthora* spp., and thereafter these workers[1,14,21] reported phytoalexin production in soybean by various treatments.

Klarman and Hammerschlag[15] demonstrated that hydroxyphaseollin was produced in association with local necrotic lesions caused by tobacco necrosis virus infection and its production quantity was proportional to the number of lesions per leaf as long as they are separated by sufficient uninfected tissue. In our experiments, hydroxyphaseollin was isolated from soybean leaves infected with leaf blight bacterium, *X. phaseoli* var. *sojae*.

We also found that its quantity in infected leaves was proportional to the number of lesions on the leaf.

We found in our antifungal bioassays that spore germ-tube growth of *Fusarium solani* f. *pisi* was severely inhibited when cultured in solutions containing hydroxyphaseollin. This inhibition of spore germination was similar to that reported for various phytoalexins.[3,13,18]

Soybean phytoalexin was detected by the TLC technique in the original work of Klarman and Gerdemann.[13] Thereafter, Klarman and Sanford[16] used it to detect the antifungal property of hydroxyphaseollin. In our experiment, crude hydroxyphaseollin was purified by the method of Keen et al[9] using TLC with hexane: ethyl acetate: methanol (60: 40: 1, v/v) solvent. The ultraviolet absorption spectrum of the ethanol extract from the Rf 0.43 zone had absorption peaks at 288, 293 and 320 nm, which were identical to the absorption spectrum of hydroxyphaseollin reported by Klarman and Sanford.[16] From these results on ultraviolet absorption spectra and inhibition of spore germination this compound was identified as hydroxyphaseollin. However, the Rf value of hydroxyphaseollin on TLC in this experiment differed from the result obtained in a similar TLC system by Keen et al.[9] A compound similar to hydroxyphaseollin was detected at the Rf 0.30 position on TLC.

The inducible production of phytoalexins is associated with localized necrotic lesions of many plants caused by fungal pathogens. However, there is little well-documented cases of a similar relationship between bacterial infections of plants and the production of phytoalexins. Stholasuta et al[23] reported that certain incompatible bacterial species stimulated production of pterocarpanoid compound, phaseollin, in bean leaves, but found that phaseollin had no antibacterial activity. Cruickshank[3] and Perrin[4,5] reported the same for phaseollin and the related compound pisatin. On the other hand, Keen and Kennedy[11] discovered that hydroxyphaseollin and related isoflavanoid compounds which accumulated in soybean leaves inoculated with *Pseudomonas glycinea* were causally related to the restriction of bacterial populations in hypersensitively responding soybean leaves and had marked antibacterial properties. The results obtained in our experiments that hydroxyphaseollin is produced in necrotic lesion of soybean leaves infected with leaf blight bacterium is identical to the results reported by Keen et al. Further selective antibacterial activity of hydroxyphaseollin should be investigated on pathogenic and nonpathogenic plant bacteria.

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摘　　要

ダイズ葉焼病罹病葉における hydroxyphaseollin の生成とその抗菌作用

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ダイズ葉焼病罹病葉からダイズのファイトアレキシンである hydroxyphaseollin が検出された.

粗 hydroxyphaseollin の量は1葉当りの病斑数と比例的で，病斑数の多い葉に多く生成された．粗 hydroxyphaseollin によって *Fusarium solani* f. *pisi* の胞子発芽，とくに発芽管の伸長が強く阻害された．Hexane: ethyl acetate: methanol (60:40:1, v/v) を展開剤とし，薄層クロマトグラフィーにより，plate 上の Rf 値0.43と0.30の部位にそれぞれ，hydroxyphaseollin とその関連物質が検出された．